

THE REPRODUCTIVE CAPACITY OF ARTEMIA SUBJECTED TO SUCCESSIVE CONTAMINATIONS WITH RADIOPHOSPHORUS ^{1,2}

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The dangers of contaminating the environment with radioisotopes or other deleterious substances have received much publicity. Often the main biological consideration has been the number of adult individuals of susceptible species seen or caught during a particular season. These numbers are then compared with records of other years in an attempt to infer damage or to claim unimpairment. Unfortunately, adult abundance equalling that of an area before exposure does not necessarily indicate recovery from genetic damage to the population. The reproductive capacity of an organism may be adequate to compensate for infecundity and the death of immature stages. Therefore, to reveal the consequences of contamination, the quantitative aspects of natality and survival to maturity must be studied.

In our laboratory, *Artemia* populations are being used in studies of induced changes in the components of biological fitness after subjection to a variety of agents. Results for the last four years of an 8-year study of radiophosphorus effects are reported below. Most notable is the consistent demonstration that the number of adults can be identical in different mass cultures, but the reproductive potential of populations with different ancestral histories differs considerably.

The fitness components obtained from *Artemia* pair mating tests over a four-year period following radioisotope exposure of ancestors were reported earlier (Grosch, 1962). Subsequently several of the original populations have been subjected to further experimentation and analyses which reveal persistent inferiority of reproductive performance in pair matings. In addition we have determined whether subpopulations can survive repeated contaminations, an important problem in radioecology. Our results indicate that species density or standing crop data can be misleading. Although adults in an experimental culture may be abundant, their reproductive potential may be inadequate to withstand further damage by radiations.

MATERIALS AND METHODS

Two isotopes, Zn⁶⁵ and P³², differing in rays, half-life, and metabolic fate, were selected for *Artemia* experiments because of their persistence in Columbia River food chains (Foster and Davis, 1955). All Zn⁶⁵ populations are now extinct, not

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only the 30 $\mu\text{c.}/3\text{L}$ culture of the 1962 (Grosch) report, but also several 20 $\mu\text{c.}/3\text{L}$ cultures followed subsequently. On the other hand, many of the P^{32} cultures survived. One of these is a 90 $\mu\text{c.}/3\text{L}$ culture which is providing useful data. In nature, although it comprises less than 1% of the radioactivity in Hanford wastes which contain more than a dozen different nuclides, P^{32} accounts for 40% to 95% of the radioactivity of most Columbia River invertebrates and fish. This reflects the biological demand for an element incorporated into genetically important nucleic acids and energy storage-transfer systems.

The three-liter mass cultures derived from the diploid amphigonic strain of *Artemia salina* have been maintained in cylindrical gallon jars for nearly a decade at room conditions in Woods Hole. During the summer they have received 1 ml. of yeast suspension daily. During the winter they typically evaporated to less than one-third of the summer volume. In spring the cultures were reconstituted by adding distilled water to dissolve salt encrustations and activate cysts. Putrefying dead algal masses were removed as soon as *Artemia* emergence seemed complete. With such attention a population of 250 to 300 adults has quickly developed in every three-liter container except in strains nearing extinction. The control cultures maintained simultaneously with experimental cultures under identical conditions, were derived from ancestors which have never been exposed to radioisotopes or other deleterious agents of technological origin.

Artemia culture techniques have been improved during the years in which jar populations have been maintained and studied. Although *Artemia* is an organism assumed to be exceptionally tolerant because the adults survive for days in a wide range of salinities, previous work (Grosch, 1962) indicated that reproductive performance was improved by increasing the salinity above that of sea water. From their own experience other geneticists (Goldschmidt, 1952; Bowen, 1962) decided to culture *Artemia* in water saltier than sea water. Prior to 1962 an increase in salinity for our mass cultures resulted only from the slow process of evaporation. Since 1962 NaCl has been added routinely to mass cultures. Present practice is to bring them to 50 grams of added NaCl per liter before maturation of the summer's first generation. In 1964 and 1965 water of increased salinity was used also for pair mating tests. Bowen's routine medium was adopted, 50 grams of NaCl per liter of filtered sea water.

Each subpopulation to be subjected to an addition of radioisotope was obtained by transferring 20 adult pairs to a gallon jar containing three liters of brine. For pair mating studies, 15 young pairs were transferred from mass culture as soon as the male had clasped the female. Since arbitrary matching was not practiced, the pairs studied are representative of those contributing to the future of the population from which they were withdrawn. Each pair was placed in its own quart jar. The average number of days between the transfer to quart jars and the death of members of mated pairs is taken as a measure of adult life span.

Jars containing parental pairs and the jars to which their broods were transferred were maintained under constant illumination from a bank of fluorescent tubes. The water temperature ranged between 25° and 28° C. All jars were examined daily at the time of feeding with yeast suspension (0.3 ml.). When present, cysts were filtered, dried and resuspended in filtered sea water for emergence tests.

RESULTS AND INTERPRETATIONS

A persistent population descended from ancestors exposed to 30 μc . of P³² produced hundreds of adults, generation after generation and year after year in mass culture. However, subcultures were unable to survive a second 30- μc . dose until four years or a minimum of 12 generations had elapsed. Furthermore, as shown in Figure 1, additional years and generations passed before subcultures managed to survive a third 30- μc . dose of P³². Evidently the carrying capacity (300 adults) of a three-liter culture was easily achieved by *Artemia* of experimental lineage. Differences between strains were revealed only by investigating reproductive performance. For this purpose we employ isolated parental pairs.

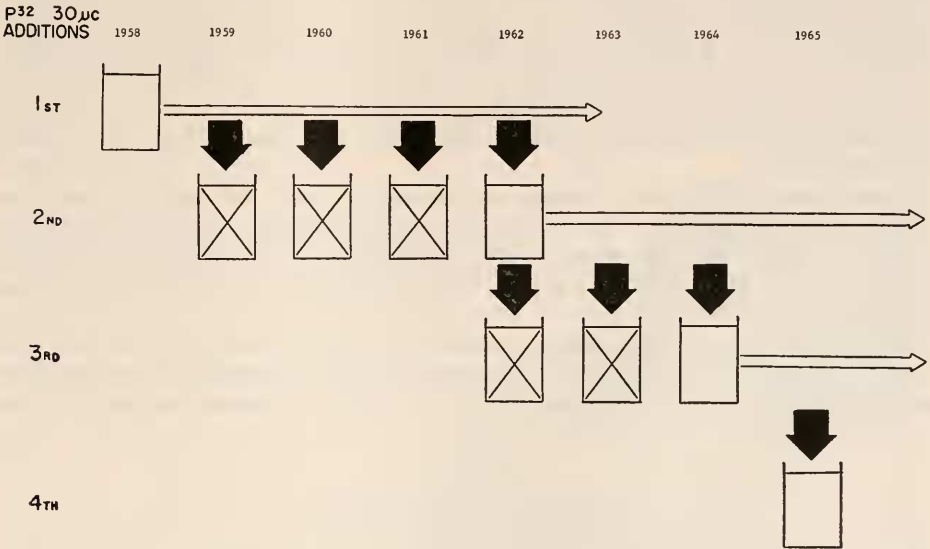


FIGURE 1. The origin and subsequent fate of three-liter experimental cultures of *Artemia* which received 30- μc . doses of P³². The black arrows indicate subcultures given radiophosphorus. Cultures marked with an X did not survive. After three months no adult offspring have appeared in the 1965 "fourth addition" jar and no horizontal arrow representing continued survival of the strain is shown. The subculture of control origin which received the first addition of P³² was discarded in 1963.

An adult female can exhibit both oviparity and viviparity (Lochhead, 1961). In pair mating tests, control females were not strongly inclined toward oviparity. They gave birth to relatively more live young and deposited fewer cysts than did females from other strains tested. Table I summarizes records for the last four years in which the percentage of zygotes encysted has been lowest for control parents year after year. In addition, for the years 1962 and 1965, the lowest percentage of emergence or "hatchability" of the cysts was found in the controls. An interesting feature of the experimental results has been the steady climb in percentage of emergence. Originally in 1959, one year after the population had experienced its first radiophosphorus exposure, emergence was less than 25%. In

TABLE I
Cyst deposition and emergence of larvae from four years' records of pair matings

Strain tested	% of zygotes encysted	1962 % emerged	% of zygotes encysted	1963 % emerged	% of zygotes encysted	1964 % emerged	% of zygotes encysted	1965 % emerged
Control	44.19	30.66	51.35	49.26	30.47	65.93	26.40	27.60
30- μ c. Experiments								
First P ³² addition	65.30	36.17						
Second P ³² addition			76.23	40.32	62.32	42.82	96.04	66.94
Third P ³² addition							87.64	63.32
90- μ c. P ³² Single dose	None		None		58.49	26.82	48.19	58.09

1965 emergence has reached a high of 66.94% for a strain which has received two doses of P³².

The results reflect the performance of all members of the sample rather than that of only a few females. Every female which produced young also yielded cysts, typically as her first brood and often as her last. This contrasts with the records in our 1962 paper in which only controls showed a majority of the females depositing cysts.

Control pairs tended to produce a greater number of broods. In addition there were more zygotes per brood and of these more survived to adulthood than survived in tests of progeny from exposed ancestors. Table II summarizes the life span

TABLE II
The life span and fecundity of parents from pair mating tests along with survival and sex ratio of offspring

	Adult life span in days		Broods per ♀	Zygotes voided		% surviving to adult	Mature adults per ♀	Sex ratio ♂/♀
	♀	♂		per brood	per ♀			
1963								
Control	16.3	14.2	1.2	272.78	327.34	29.91	97.91	0.97
30- μ c. second addition	16.6	21.9	1.4	130.70	182.98	12.85	23.51	0.95
90- μ c. single dose	12.7	12.8	0.3	74.33	22.30	0.58	0.13	1.33
1964								
Control	60.45	66.65	12.25	187.35	2295.04	73.49	1686.62	0.80
30- μ c. second addition	33.62	44.38	2.87	133.98	384.52	49.93	181.99	0.88
90- μ c. single dose	30.60	31.60	5.7	177.84	1013.68	17.07	173.35	0.99
1965								
Control	55.45	52.18	10.18	157.17	1599.99	59.45	951.18	0.85
30- μ c. second addition	21.92	31.17	3.83	106.26	406.96	47.70	194.12	0.91
30- μ c. third addition	30.71	36.71	4.36	109.59	477.81	56.71	270.97	0.81
90- μ c. single dose	32.08	38.00	3.63	81.45	295.66	68.71	203.15	0.90

of parents and their reproductive performance for 1963 through 1965. Included are the sex ratios for adult progeny. Females are favored, except in 1963 for the 90- μ c. culture which was then not doing well. These results differ from the earlier sex ratios which favored males in many irradiated strains (Grosch, 1962).

Sex ratio can be discounted but the problem is to decide if improvement in any other aspect such as adaptive value is involved in surviving an additional radioisotope contamination. Adaptive value can be defined as the relative capacity of carriers of a given genotype to transmit their genes to the gene pool of the following generation. It may be calculated by dividing the mature adults per pair of treated ancestry by the number of mature adults per control pair. The adaptive values for the 90- μ c. strain have shown regular improvement from nearly zero in 1962 through 0.001, 0.10, to 0.21 in the last three years. On the other hand adaptive values have varied for the 30- μ c. doses, due partly to variability in control performance which is taken as unity in deriving the value. On this basis an average of 191.99 live adults in 1964 does not compare so well with 194.1 in 1965 when respective control performance is used as the basis for calculating A.V.'s of 0.11 and 0.20. Possibly prolonged patterns of weather are reflected in our data; 1964 and 1965 were different types of summers.

The reproductive advantage of controls, expressed as mature adults produced per parental pair, depends in part upon the length of time adults survive in quart jars. In 1963 before the Bowen medium was adopted for pair mating tests, the ratio of control to "second addition" progeny, 97.9:23.5, was about 4:1. In 1964 the ratio was about 9:1 and in 1965 the ratio was about 5:1. Part of the difference comes from an increase in survival (mean adult life span) from less than three weeks to approximately two months. Although the poorest experimental groups are now attaining life spans equivalent to or exceeding control values of previous years, the difference which was not apparent in 1963 has become pronounced. Controls are now living nearly twice as long as adults from experimental populations.

With increased life span, improvement in the number of offspring per female was inevitable because broods have been deposited with regular frequency. The raw data are too extensive for tabulation here. Analysis of 1965 brood frequency showed no significant difference in the averages of the interval for control (3.00 ± 0.91), second (3.20 ± 0.27), and third addition (3.80 ± 0.94) tests. This pattern in which females void broods every third or fourth day has been evident in our records since 1959. In 1963 when pair matings were still maintained in ordinary sea water the control interval was 3.86 ± 0.06 days and for second addition pairs 3.25 ± 0.30 days. A striking and unexplained deviation in the pattern has appeared for the 90- μ c. strain. In 1964 the interval averaged 3.07 ± 0.02 days. In 1965 the strain showed a longer period between broods; the interval of 6.80 ± 0.98 days differs significantly from all others cited. These frequencies which concern only the pattern between the first and last brood voided cannot be inferred directly from the number of broods per female shown on Table II. The time elapsing between isolation of pairs and deposit of first brood varies as does the period intervening between last brood and death.

Another influence of life span appears to be upon brood size but the relationship is not simple. In young adults brood size is correlated with the increasing size of

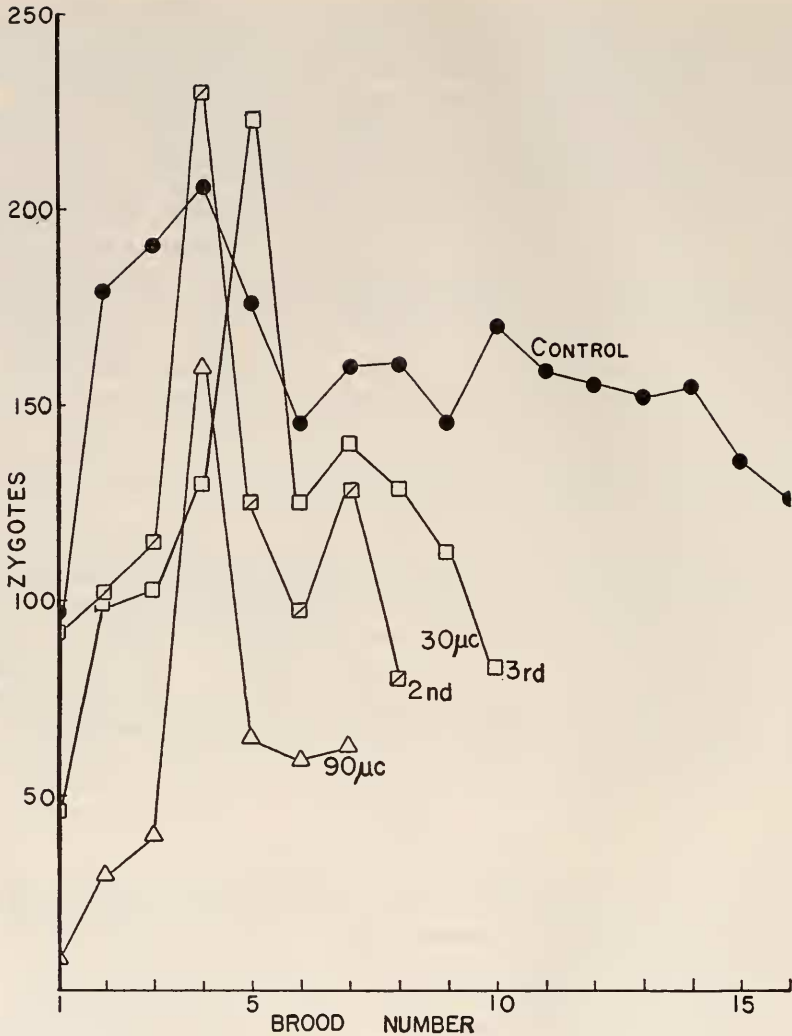


FIGURE 2. Brood size plotted against the sequence of brood deposit. Each curve represents the reproductive record of the individual female surviving for the longest period of time in its respective group of 1965 pair mating tests. The solid circles indicate the control example. Triangles represent values for the long-lived individual from a culture which received a single 90- μ c. dose of P^{32} in 1959. Squares mark values for the examples from 30- μ c. addition experiments. The number of successive doses in the history of the culture is indicated by a designation at the end of the curve, 2nd = 2 and 3rd = 3 doses.

the female. Subsequently brood size declines, presumably as a reflection of aging or because of debility associated with impending death. The clearest demonstration of this pattern was obtained by plotting brood size against brood sequence for each individual female. A peak and subsequent decline is characteristic of records for all females surviving 15 days, the time usually required for voiding about five

broods. Plotting the average brood size is an unsatisfactory procedure in this case. Particularly for experimental females the pattern of fecundity tends to be obscured in pooled data by the broods contributed by short-lived females.

In order to reveal potentialities in fecundity, patterns of brood size for females of maximum longevity from each of the populations sampled by pair mating tests in 1965 are shown in Figure 2. Peak productivity was achieved by the fifth or sixth brood. The subsequent decline was pronounced for females of experimental ancestry but moderated by a long plateau for the control sample. As evidence that the example is representative of the control pattern we can state that 7 of the first 10 points are nearly identical with the respective average values, and the remaining three points lie within one standard error of the pertinent control values. The control sample which averaged 10 broods before death (Table II) contained many individuals which survived beyond the period of maximum fecundity. On the other hand, experimental females which averaged only 3 or 4 broods before death probably failed to reach their maximum fecundity.

Although deficient, the fecundity of "second addition" pairs has not varied in a manner which would explain the response of the mass culture to additional isotope exposures. A possible key may be the survival to adulthood. These percentage values fit into a pattern:

Year	1959	1960	1961	1962	1963	1964	1965	Addition
Survival	24.4	27.6	57.6	61.0				First
					12.8	49.9	47.7	Second

Not until 1962 was a second addition tolerated. Survival to adulthood was reduced severely by the treatment and a third addition was not tolerated until the moderate recovery demonstrated in 1964 had occurred.

DISCUSSION

A twice-repeated series of P³² doses had indicated that a single addition of 90 μ c. approached the threshold dose for population extinction (Grosch, 1962). From the simple standpoint of dose arithmetic it seemed possible that subcultures from a strain that had survived 30 μ c. should be able to withstand additional doses. This was not the case, and pair mating studies revealed severe genetic damage to reproductive ability from one 30- μ c. treatment. Additional equivalent doses were tolerated only after a period during which the germ plasm presumably underwent a partial purge.

The pair mating tests summarized above reveal that maintenance of populations numbering 300 adults amounts to only a small fraction of the control potential. For example, if 300 adults comprise 150 pairs each capable of providing 951 mature offspring (1965 average), the potential number of offspring amounts to the product, 142,650. However, when the carrying capacity determined by a density-dependent process is 300, only a fraction of the reproductive potential can be utilized. This fraction $300/142,650 = 0.002$ or 0.2%. By the same reasoning, only 1% of the "second addition" culture's potential is required to maintain the ceiling level of 300 in a three-liter culture. In the past, a number of cases was observed in which 10% to 30% of the reproductive potential was used to maintain 300 adults in a

three-liter culture. This type of situation proved precarious and many such cultures became extinct. Conceivably even more extreme situations may occur in nature, and in unfavorable circumstances 80% to 100% of the reproductive potential of an organism may be required to maintain the frequencies of adults found in census.

As pointed out by Grant (1963), we don't really have enough quantitative information at present about the actual number of genetic deaths a population can tolerate and still survive under various conditions. Possibly more than 50% of the *Artemia* reproductive potential must be held in reserve to buffer populations against extinction. If so, in natural situations impaired by human activities, other organisms may be balanced even more precariously than *Artemia*. Actually *Artemia* may have an advantage over most animals. Possibly when shrimp populations reach the ceiling level their excess productivity can be switched into encysted zygotes rather than expended in juvenile mortality.

Fertility requirements are very high, usually too high, for a species acquiring many deleterious two-allele loci with high selective differentials. Several geneticists (Lerner, 1958; Wallace and Dobzhansky, 1959; Grant, 1963; Wallace, 1963) have speculated about the number of offspring which must survive if a population is to avoid extinction following the induction of simple dominant or recessive lethals. The maximum number of offspring is limited by the number of functional eggs produced per female. In this attribute *Artemia* exceeds domestic animals and most insects. On the other hand, *Artemia* fecundity is not particularly exceptional when compared with the range for fish and aquatic invertebrates (Altman and Dittmer, 1962). The cost in segregation of inferior homozygotes may be met by a fecund organism, or the price may be reduced by series of multiple alleles or by numbers of independently assorting interchangeable genes (Grant, 1963).

An alternate approach is to view lethality as the product of lethal gene combinations rather than the product of lethal genes (Mayr, 1963). The genes which interact harmoniously in the population's gene pool were brought together by natural selection acting over a long period of time. Disharmonious combinations can follow the induction of genic diversity. Recently bichromosomal synthetic semilethals have been demonstrated in *Drosophila pseudoobscura* (Dobzhansky *et al.*, 1965). Individuals homozygous for specific second and third chromosomes showed viability down in the semilethal range. Conceivably disharmonious interactions also can occur in heterozygous genotypes. So many beneficial effects of heterozygosity have been described that we are too inclined to regard all heterozygosity as good, but for example, a loss of epistatic balance among interacting loci can override the beneficial effect of high heterozygosity (Mayr, 1963).

To date we have been unable to demonstrate increased genetic fitness in irradiated *Artemia* such as shown by Wallace (1956) in *Drosophila* and by Crenshaw (1965) in *Tribolium*, but admittedly a demonstration of the phenomenon may require a more inbred strain of shrimp than is yet available. Also our testing has been limited to a particular season. Possibly in certain seasons (or years) it might be possible for experimental *Artemia* to equal or exceed the controls in fitness. On the other hand, Sokal and Huber (1963) reported heterozygote intolerance to crowding in one *Tribolium* experiment, and Sankaranarayanan (1965) found *Drosophila* subpopulations plateauing at 70 to 75% viability in x-ray experiments. This level achieved in five generations after cessation of irradiation

indicated more rapid recovery than we have observed with *Artemia*, but gives no evidence of superiority of irradiated strains.

In prolific mass cultures, crowding is more pronounced than that experienced by broods in quart jars. Until this year survival to adulthood in experimental broods in quart tests has differed obviously from control values, and any effect of crowding has been obscured. However, even the 90- μ c. strain has improved to the degree that its fewer zygotes per brood (81.45) enable compensation in a higher percentage of survival to adulthood in the 1965 results of Table II. When 68.71% survival was compared with the control's 59.45%, the contingency chi square calculated from the raw data was 11.48 with $P < 0.001$. On the other hand, 106 to 109 shrimp per quart jar do not provide a situation significantly different from the control's 157 per quart. In these cases survival to adulthood does not exceed that of controls.

Fly crowding experiments furnish some interesting parallels and differences. Survivorship near 60% can be demonstrated through a wide range of fly densities for a variety of strains, and in house fly experiments Sullivan and Sokal (1963) quote 67% survivorship for a density of 160 per bottle, considered "normal conditions." Competition became impressive only at 1280 flies per bottle and extreme at 2560 per bottle. This was reflected by "negligible" adult emergence, 1% or less in some strains (Bhalla and Sokal, 1964). Thus normal density in fly cultures gives survival similar to that in *Artemia* brood tests, and high density fly experiments more nearly resemble what may happen in prolific mass cultures of shrimp. Moderate crowding to give selection pressure of intermediate intensity of *Tribolium* provides another example of insect survivorship within a 58 to 78% range (Sokal and Huber, 1963).

Differences from insect results derive from differences in growth pattern and become pronounced as development nears maturity. Size is determinate for holometabolous insects and indeterminate for shrimp. In crowded dipteran populations the usual response is a maintenance of numbers accompanied by a reduction in size of individuals (Sullivan and Sokal, 1963). Brine shrimp respond by repressed rate of growth and delayed maturity of part of the group or brood (Grosch, 1962), suggesting a feedback phenomenon such as reported by Rose (1960) for fish and Amphibia. Unfortunately, for purposes of comparison, cannibalism occurs among crowded fish.

If adults function as growth suppressors, their longevity could interfere with a turnover of generations. No data are available on the effects of crowding on the longevity of *Artemia* adults but with moderate fecundity and good potential survival to adulthood, parents need live only long enough to produce a brood or two in order to maintain a mass population in three liters. Our life span data concern isolated pairs. Although present techniques prolong adult survival, individual examples of extreme longevity, such as reported by Lochhead (1941) and Bowen (1962), have not been obtained. However, frequent transfer is necessary in order to assess the reproductive performance of pairs and under such circumstances even inanimate objects like cafeteria tumblers have a predictably limited life span (Brown and Flood, 1947).

SUMMARY

1. This paper is a progress report on four additional years of studying *Artemia* in and from mass cultures to which radioisotope has been added.

2. Although the number of adults seen in mass cultures may be equivalent, subcultures of control and experimental strains react differently to radioisotope additions. Strains descended from ancestors exposed to P^{32} do not necessarily survive a second dose even though total dosage does not exceed the extinction dose given as a single addition. A period of recovery involving a passing of generations must intervene. Depending upon culture conditions, this may involve two to four years.

3. Pair mating tests revealed that in comparison with controls, experimental strains:

- A. Have a shorter life span.
- B. Deposit fewer zygotes per brood.
- C. Deposit more of their developed zygotes as viable cysts.
- D. Show poor survival to adulthood except when crowding is mitigated by low fecundity.

4. Because the frequency of brood deposit has been regular in both control and experimental strains, an increase in total progeny has accompanied increased life span. The one exception to an interval of three to four days between deposits occurred in 1965 records of a strain recovering from a near lethal dose.

5. The sex ratios among adult progeny from pair matings now favor females in all strains.

6. On the basis of pair mating tests, maintenance of mass cultures at an observed level of 300 adults per three liters requires only 0.2% of the reproductive potential of controls. Cultures of experimental origin utilize 1% or more of their potential to maintain the same total.

7. The proportional number of larvae surviving to adults may be critical in determining whether or not a strain can tolerate another exposure to a radioisotope.

8. Although general comparisons may be drawn to crowding experiments in insect populations, a complete parallel is impossible because of the shrimp's indeterminate growth pattern (although *Artemia* populations are inversely density-dependent).

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